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Surveillance and Comparison of Anaplasma Phagoctyophilum (Formerly Erlichia Equi) within Ixodes Scapularis Ticks in Selected Southeastern States

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SURVEILLANCE AND COMPARISON OF *ANAPLASMA PHAGOCYTOPHILUM* (FORMERLY *EHRLICHIA EQUI*) WITHIN *IXODES SCAPULARIS* TICKS IN SELECTED SOUTHEASTERN STATES

by

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(Under the Direction of Quentin Q. Fang)

ABSTRACT

Anaplasma phagocytophilum is an obligate intracellular bacterium that can infect and cause disease in horses, Equine Granulocytic Anaplasmosis. The bacterium is present in the western and northeastern United States, Europe, and Asia. In this investigation, samples of *Ixodes scapularis* were collected from selected barrier islands and mainland sites where feral and domestic equine populations are present, respectively. Each sample was individually screened using nested PCR to amplify a fragment of the *ank* and 16S rRNA genes. The prevalence of *A. phagocytophilum* in *I. scapularis* ticks was 20% (n= 808). The highest infection rate was seen at a barrier island site (22%, n= 774). The remaining five sites had prevalence ranging from 0% (n= 9) to 19% (n= 51). Randomly chosen positive PCR samples from each site were sequenced, and a BLAST search verified the isolates as *A. phagocytophilum*, which shared sequence homology with isolates from the northeastern United States.

INDEX WORDS: *Anaplasma phagocytophilum*, granulocytic Anaplasmosis, *Ixodes scapularis*, tick-borne disease

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CHAPTER 1

INTRODUCTION

Rickettsiae and the pathogen *A. phagocytophilum* (formerly *E. equi)*

Anaplasma phagocytophilum is a rickettsial bacterium that is classified within the family Anaplasmataceae based on its morphology and development. This bacterium is Gram-negative with a coccoid or pleomorphic rod shape. Like all ehrlichiae, *A. phagocytophilum* invades host white blood cells, specifically the neutrophils and eosinophils in equine infection. The developmental cycle of the obligate intracellular bacterium is comprised of three stages, of which the final stage is used for identification of the genus (Sonenshine, 1993). The infective stage, the elementary bodies, enters the host cell by phagocytosis. Within the phagosomes, the elementary bodies replicate by binary fission and aggregate into clusters called initial bodies. The initial bodies further grow and replicate into morulae, which look like bunches of grapes and can easily be seen by microscopy. In comparison to morulae of other ehrlichial species, *A. phagocytophilum* morulae appear as loose aggregates of individual organisms that contain a cell wall and membrane and are confined to membrane-lined cytoplasmic vacuoles within the host cells (Lewis, 1976).

The invasion of a host's white blood cells leads to an infection called equine anaplasmosis or equine granulocytic anaplasmosis (EGA). Horses infected with *A. phagocytophilum* present characteristic symptoms that include fever, limb edema, uncoordination, petechiation, anorexia, depression, icterus (=jaundice), ataxia, and hypophagia (Madigan, 1993). Hematological findings show signs typical of immunosuppressive infections, such as thrombocytopenia, leucopenia, decreased packed

cell volume, and elevated plasma icterus indices (Gribble, 1969). Additionally, blood smears show the presence of the inclusion bodies known as morulae in the cytoplasm of the infected neutrophils and eosinophils.

The first recorded case of EGA occurred in 1961 in northern California, followed by three more infections that eventually led to its official description and report by Gribble (1969). The majority of *A. phagocytophilum* infections in horses have been in western states, mainly California, with only sporadic cases occurring in the eastern United States (Madigan & Gribble, 1987). While individual reports of the disease have appeared in Florida and New Jersey (Madigan & Gribble, 1987), the first diagnosed case on the eastern coast occurred in 1994 in New England (Madigan, 1996) where a localized epizootic of equine anaplasmosis affected over 50 horses. The index case was a Quarter horse-cross gelding exhibiting symptoms of infection such as lethargy, fever, limb edema, petechiation, and thrombocytopenia. A blood sample was analyzed using the nested PCR utilized to diagnose horses in California. The result was positive, and following 16S rRNA gene sequencing it appeared the species was more similar to the agent of human granulocytic anaplasmosis (HGA) than California horse isolates of *Anaplasma phagocytophilum*. Interestingly, the cases were seen in a region with high prevalence rates of the human granulocytic anaplasmosis agent. In the paper in which the study was presented, four other cases were observed that showed positive sample analysis. The importance of this study is, firstly, the confirmation of a disease that was thought to be limited to the west coast. Secondly, the similarity in sequence to the HGA agent suggests humans could become infected with the EGA agent if fed on by an infected vector.

Equine anaplasmosis cases were also seen in Sweden starting in 1995 (Pusterla et al., 1998). In 1998, pathogenesis of anaplasmosis in a 12 year old gelding was facilitated by immunosuppression resultant from a colic operation (Pusterla et al., 1998). Clinical symptoms were of the early stages of disease, and confirmation of *Anaplasma phagocytophilum* infection with PCR also demonstrated the homology of the isolate with the human granulocytic anaplasmosis agent in the United States and dog and horse granulocytic anaplasmosis causative agents previously identified in Sweden (Johansson et al., 1995). The manifestation of the disease in the above case demonstrates the weakening effect *A. phagocytophilum* infection has on the host immune system as a result of neutrophil and eosinophil invasion. Thus, immunocompromised individuals are more susceptible to the development of equine anaplasmosis upon infection with the causative agent.

Manifestations of anaplasmosis are associated with a reduction of the leukocytes due to infection with *A. phagocytophilum* and additional immunosuppression caused by age, illness, or drug therapies. However, the host range for *A. phagocytophilum* infection alone does not have such limitations. Experimental inoculations of sheep, goats, and dogs with *A. phagocytophilum* previously showed susceptibility of mammals to infection and mild disease (Stannard et al., 1969).

Confirmation of previous findings, in addition to susceptibility of other mammals, was completed by Lewis et al (1975). Beagle and German Shepard dogs, domesticated cats, macaques, baboons, white mice, golden hamsters, guinea pigs, and New Zealand white rabbits were inoculated with *A. phagocytophilum*- infected equine blood. The findings showed that canids, felids, and non-human primates can present signs of

infection, including morulae in the eosinophils and/or neutrophils. Thus, *A. phagocytophilum* infection is not species-specific, and the susceptibility of non-human primates elucidates the vulnerability of humans to infection.

The discovery of a human ehrlichial agent in 1994 further supported previously speculated human infection (Chen et al., 1994). The blood of individuals from Minnesota and Wisconsin presenting febrile symptoms was tested by PCR using universal primers based on *Ehrlichia* species. A human ehrlichial species was identified with similarity to *Ehrlichia phagocytophila* and *E. equi* 16S rRNA sequences. In a more comprehensive study, isolates of *A. phagocytophilum* (= *E. equi*) in horses and the HGA agent in humans were analyzed for sequence variations in the 16S rRNA, 444 *Ep*-ank, and *groESL* genes (Chae et al., 2000). Researchers found the 16S rRNA gene in the ehrlichial species to be either identical or with as little as one nucleotide difference. Any slight variations in the gene sequences were attributed to the geographic origin of the isolate. The demonstration of sequence homology between the EGA agent and the causative agent of human anaplasmosis, *Anaplasma phagocytophilum*, suggests a close phylogenetic relationship between the organisms.

While sequence homology between the EGA agent and *A. phagocytophilum* was established, the two species were still considered independently until the presentation of cross-reactive serological findings. Experimental inoculations of sero-negative horses with *A. phagocytophilum* have previously been successful, with the inoculated horses presenting clinical symptoms, pathology, and etiology typical of equine anaplasmosis (Madigan et al 1995, 1996, Chang et al. 1998, Pusterla et al 1999). Additionally, a sero-negative horse inoculated with *A. phagocytophilum*, the HGA agent, was protected

against infection with the EGA agent (Barlough et al, 1995). The result of the challenge was PCR negativity, a lack of clinical symptoms, and increases in serum antibodies, thus suggesting the initial inoculation protected against infection by the second ehrlichial species. These results indicate that the HGA agent and EGA agent (*E. equi*) share significant antigenicity.

Working from previous findings that individuals diagnosed with HGA develop antibody resistance to *Ehrlichia equi* and *Anaplasma phagocytophilum*, researchers developed a serological cross-reactive indirect fluorescent-antibody assay (IFA) protocol to support the hypothesis (Dumler et al., 1995). The sera of horses naturally infected with *Ehrlichia equi* was not tested for reactivity with *Anaplasma phagocytophilum* antibodies by IFA, but sera from humans infected with the HGA agent was strongly reactive with *Ehrlichia equi* antibodies by IFA. Broad cross-reactivity between the two ehrlichial species of interest demonstrates a greater similarity beyond sequence homologies, and the different species may actually represent a single species. The significance of demonstrating the single species hypothesis is important in the distribution of equine anaplasmosis. The demonstration of "ehrlichia of human origin as a cause of EGE under experimental conditions…suggests…the agent may be responsible for field cases of EGE as well, particularly in areas of the country where EGE is prevalent" (Madigan et al., 1996). Thus, the distribution and prevalence of the HGA agent should parallel the distribution of the HGA agent. In 2001, Dumler et al. suggested the designation of *A. phagocytophilum* and *E. equi* as synonyms. Therefore, in the remainder of the investigation, the two may be used interchangeably depending on the infected host and historical reference.

It has only been recently that surveillance studies for *A. phagocytophilum* have been conducted. In 2002, coastal sites in South Carolina, Georgia, and Florida were used for collection of ixodid ticks to test for HGA (Fang et al., 2002). The findings of the study showed occurrence rates ranging from 0% to 20% at individual sites, and an overall occurrence rate of 1.6%. The study shows the potential for *A. phagocytophilum* incidences in the southeastern United States, and the need for further studies to more thoroughly assess the prevalence of this rickettsial bacterium.

The tick vector *Ixodes scapularis*

The transmission of *Ehrlichia equi* (=*A. phagocytophilum*) to susceptible equids was speculated to involve ticks as vectors of the bacteria since the causative agent's first description by Gribble (Lewis, 1976). The first experimental attempts to transmit bacteria utilizing engorged nymphs from *Ehrlichia equi*-infected horses to sero-negative horses were unsuccessful because the tick species used, *Dermacentor occidentalis* ("Pacific Coast tick"), is not a competent host for the organism (Gribble, 1969). It was still believed that infection was facilitated via an arthropod vector because of the bacteria's similarity to other tick-transmitted causative agents; instead, it was proposed that infected horses are not effective reservoirs of *Ehrlichia equi* (Lewis, 1976).

A significant breakthrough demonstrated that *Ixodes pacificus* ticks are vectors of *Ehrlichia equi* on the western coast of the United States (Richter et al., 1996). Ticks within the same genus were proposed as vectors for transmission in the eastern United States (Madigan et al., 1996). Confirmation of *Ixodes scapularis* as the vector in this region was achieved with the successful isolation of *Ehrlichia equi* in tick cell culture

(Munderloh et al., 1996). The distribution of the vector extends into the region of interest in the current investigation; suggesting the presence of *Ehrlichia equi*.

Molecular and microbiology techniques for detection

The first studies of equine anaplasmosis were conducted in the 1960s when microscopy techniques were utilized to identify the causative agent. In early investigations, simple staining techniques such as Gram, Giemsa, and Wright-Leishman stains (Stannard et al., 1969) were applied to identify morulae within blood smears, and electron microscopy was also employed to examine infected leukocytes (Gribble, 1969). While these methods are still time and cost efficient, more advanced molecular techniques have been developed to identify *Ehrlichia equi* in hosts.

Advanced immunological techniques such as indirect fluorescent-antibody assay (IFA) have been developed and utilized for *Ehrlichia equi* (Nyindo et al., 1978). The immunoassay employed a slide coated with the antigen using infected neutrophils. An unlabeled specific antibody reacted with the target antigen, followed by the reaction of fluorescently labeled anti-immunoglobulin as the secondary antibody. Western blotting has also been used for pathogen detection, where denatured antigen proteins are separated based on their mass, transferred to a nitrocellulose sheet, and bind to applied antibodies that allow the protein bands to be detected.

Other molecular techniques are required to detect *Ehrlichia equi* in tick vectors but can also be used with equine host samples. Polymerase chain reactions using primers based on eubacteria gene sequences have been used to amplify specific sequences of the bacterial DNA. The PCR technique was first developed by Kary Mullis in the late 1980s (Eeles and Stamps, 1993). The oligonucleotide RNA primers anneal to the denatured

Ehrlichia equi template and *Taq* polymerase extends the gene of interest; the process is repeated in a number of cycles until the gene is increased 10^6 to 10^8 fold. Gel electrophoresis is then performed to visualize the band of interest, which is identified by the position on the gel compared to a standard, indicating the number of nucleotide bases in the gene. A nested polymerase chain reaction, involving two primer pairs, for detecting the 16S rRNA gene of *Ehrlichia equi* in horses and ticks was developed by Barlough et al (1995). Additional gene sequences have been amplified for greater specificity (Massung et al., 1998) and to determine the phylogenetic relationship of *Ehrlichia equi* with other rickettsial bacteria.

Screening

The current investigation sought to detect *A. phagocytophilum* (formerly *E. equi*) DNA in ixodid tick samples. Therefore, immunological assays and microscopy methods were not utilized. Polymerase chain reactions were conducted to amplify two genes of the infectious agent, one with low phylogenetic diversity and one with high phylogenetic diversity and specificity. The goal of the PCR was to determine positive or negative presence of the bacteria; therefore, a nested PCR for qualitative data was performed instead of a Real-Time PCR that provides quantitative data.

Primer design is perhaps the most important component in molecular and microbiology research. Without the proper primers, the target region of a gene cannot be amplified, and thus the results of a polymerase chain reaction are inconclusive.

Based on the rules put forth by Innis and Gelfand, the most successful primers are 18 to 28 nucleotides long (1991). Additionally, the composition of the primer has certain parameters that increase its annealing properties. The base composition should be 50-

60% G and C, the 3' end of the oligonucleotide primer should be CG, GC, C, or G, and runs of three or more Cs or Gs at the 3' end should be avoided. Primers that have been designed based on the above parameters may still fail if secondary structures form, because of self-complementation, or annealing temperatures are less than 5°C below the melting temperature of the primer. When developing primers based on protein gene sequences, the amino acid sequence should also be analyzed to determine if the primers change the amino acid sequence by displacing codons, and thus coding, of the region amplified. Primer sequences from previous research (Bjöersdorff et al., 2002, Barlough et al, 1996) were used to amplify the desired region of the genes of interest.

The genes that were used in the *A. phagocytophilum* investigation include the 16S rRNA gene and a protein gene called *ank*. The 16S rRNA gene is commonly used in PCR techniques because of its short length and low background product. Since there is very little phylogenetic diversity within the highly conserved gene, an additional primer set based on a different amplicon must be used to specifically identify *A. phagocytophilum* within the samples. Several studies have been conducted to compare the detection of *Ehrlichia phagocytophila* group sequences using primers based on different genes. The most common genes used were *groESL* or *groE*, *msp2*, and *ank*. Of these, *groESL* was found to show little phylogenetic diversity between aligned sequences and amplification was weak (Caturegli et al., 2000 and Massung et al., 2003). The results of *msp2* analysis showed high specificity and low background product (Massung et al., 2003). The *ank* gene also showed high specificity (Massung et al., 2003). Based on the studies and the sequences available for alignment, the protein gene *ank* was chosen to supplement the 16S rRNA gene primers.

AnkA is a tandemly repeating cytoplasmic protein antigen. The *ank* gene fragment that encodes the 160 kDa protein is 444-bp long (Caturegli et al., 2000). The *A. phagocytophilum* sequence of the gene was found to have an 81-bp deletion at nucleotide position 2828, distinguishing it from the *ank* gene of other ehrlichial species (Massung et al., 2000).

Significance and Objectives

Previous cases of *A. phagocytophilum* infections have found the vector-borne disease in domestic horse populations in the United States, primarily along the northeastern and western coasts (Madigan et al., 1987, 1996; Gribble, 1969). Despite reports of equine anaplasmosis within the region (Brewer, 1984), no research has been conducted to detect the prevalence of the bacterial agent in horse populations, either domestic or feral, and tick populations in the southeastern United States. More sampling must be done to accurately contrast the infection rates to those found in the northeast, where many studies have already been conducted. The current investigation was a surveillance of *A. phagocytophilum* within the ixodid tick populations of the southeastern United States to determine infection susceptibility of feral and domestic horse populations in the region.

The results of the research will be significant in the monitoring of native and non-native wildlife, specifically feral horses, on the sites surveyed. Positive samples will indicate to the National Park Service at Cumberland Island and Shackleford Banks that monitoring of the horse populations is necessary to maintain the health of feral horses on the island. It will also indicate in all areas that surveillance of ehrlichial species must be performed to protect the public who visit those parks, owners of domestic horses, and

wildlife from infection by the bacteria. Owners of domestic horses in the research areas will be informed of the possible risks posed by positive evidence of *Ehrlichia* and steps they may take, with the aid of veterinarians, to survey the prevalence of the agent in their horses.

The primary objective of the study was to infer the prevalence of *A. phagocytophilum* in horse populations based on DNA detection in tick samples collected at domestic and feral horse habitats including Bulloch, Camden, Glynn, and Chatham counties and Cumberland Island in Georgia and Shackleford Banks in North Carolina. The sampling was performed to contrast the infection rates to those found in the Northeast. Additionally, the infection rates in tick populations near feral horses were compared to those of tick populations near domestic horses.

It was hypothesized that the isolation of tick populations on the barrier islands, Cumberland Island and Shackleford Banks, would facilitate an increased density of *A. phagocytophilum*-infected ixodid ticks. It was also hypothesized that a significant number of tick samples would be PCR- positive for *A. phagocytophilum*. The basis for the hypothesis was the presence of the primary reservoir/host and tick species in the region and infection rates for the genetically homologous *Anaplasma phagocytophilum* in previous studies. In addition to the main objective, the following questions will also be addressed at the completion of the study:

Do the collected ticks carry *A. phagocytophilum*?

Could any feral horses be infected with *A. phagocytophilum*?

Are domestic horses in Georgia and North Carolina at risk for infection with *A. phagocytophilum*?

Does the DNA sequence of *A. phagocytophilum* detected differ from that of other regions (Western United States and Europe)?

Do infection rates of *A. phagocytophilum* vary from infection rates in other regions?

CHAPTER 2

MATERIALS & METHODS

Population

Two sample units were analyzed, including ixodid ticks collected from the vegetation on Cumberland Island and Cape Lookout, which were classified as feral horse population sites, and ixodid ticks collected from domestic horse pastures in southeastern Georgia, which were classified as domestic horse population sites. Based on morphology, the ixodid tick populations collected for the study consisted of only *Ixodes scapularis*.

Study sites

The study sites included Cape Lookout National Seashore in North Carolina, Cumberland Island National Seashore in Georgia, and private property of consenting horse owners in Camden, Glynn, Chatham, and Bulloch counties in Georgia. These sites were randomly chosen based on accessibility and low levels of urban development. Vegetation areas on Cumberland Island and Cape Lookout were chosen based on dense vegetation, proximity to the horses, and distance from roads, if present. Vegetation areas with domestic horses were chosen based on grazing areas on the landowners' properties, vegetation density, and frequency of trail use.

Cumberland Island National Seashore is an extremely diverse ecosystem in southeast Georgia rich with shore birds, feral horses, introduced armadillos and wild pigs, and sea turtles that thrive within the beaches, marshes, and maritime forests. Tick collections on the island took place in areas rich in woody vegetation within the maritime forests. Since the island is large, approximately 18 miles long, the collecting sites were

randomly chosen within 3 areas of the island: northern, central, and southern. Collection areas were divided into zones A through F at the southern region, zones G through I at the central region, and zones J through L at the northern region. The location of the zones is indicated in Figure 2.1.

Cape Lookout National Seashore is comprised of the study site Shackleford Banks, a barrier island off the coast of North Carolina. The island sustains a monitored feral population of approximately 128 horses, in addition to other fauna and flora. The island is smaller in comparison to Cumberland Island and has smaller areas of maritime forest; thus, the collection site was not divided into areas or zones. Ticks were collected from the maritime forest on the western end of the island, where no roads or signs of human disturbance were present. The lined area in Figure 2.2 represents the collection site on Shackleford Banks.

Areas that were dragged for ticks on private properties were winter pastures, where more woody vegetation is prevalent, and trails used for exercising horses. The size of the areas varied depending on land ownership. In Camden, Bulloch, Chatham, and Glynn Counties, trails in a 1 to 2-mi^2 area were dragged during the second winter collecting season of the study (Fig. 2.3 to 2.6). The site in Camden County was located near the horse community, Plantation Point (30° 57’ N; 81° 44’ W). The forest was comprised of oak and cypress trees. In Chatham County, the collection site was located near Cutter Ridge Ranch in Rincon, GA (32° 12’ N; 81° 14’ W). Collection in Bulloch County was performed at Evermore Farm in Brooklet, GA (32° 23’ 19.41’’ N; 81° 32’ 40.3656’’ W). Collection in Glynn County took place in Paulks Pasture Wildlife Management Area (31° 8’ 34.3572’’ N; 81° 28’ W). The forest of Chatham, Bulloch,

and Glynn Counties were comprised of longleaf-slash pine with wiregrass and briar understory.

Sample collection

A combined total of 808 ixodid ticks were collected from the sample site vegetation. Six-hundred and thirty three samples were collected from Cumberland Island, 43 from Cape Lookout/Shackleford Banks, 55 from Camden County, 44 from Bulloch County, 24 from Chatham County, and 9 from Glynn County.

Tick collection

Ticks were collected from vegetation using a 1 by 1-m drag cloth and transferred as collection groups into the tissue preservative, RNAlater® (Qiagen, Valencia, CA). Collection groups included no more than 25 ticks per tube, and each tube was numbered and identified with site location. To perform the sweep, the drag cloth was moved across vegetation multiple times to increase tick quantities. *Ixodes scapularis* ticks were removed from the cloth and transferred to storage tubes using forceps.

DNA Isolation

Tick samples collected were removed from the collection tubes, dried, and individually separated at the lab facilities into 1.5 mL microcentrifuge tubes for DNA isolation. DNA isolation was achieved using a modified version of the Master Pure (Epicentre® biotechnologies, Madison, WI) nucleic acid isolation protocol for samples EE1-EE423. First, 300 µL of 2X Tissue and Cell Lysate Solution and 1.0 µL of Proteinase K were added to each tick sample-tube. The tick tissue was homogenized within the tube using a pestle. Once the ticks and solution were well homogenized, each sample was briefly vortexed. Next, the samples were incubated at 65°C for 20 minutes,

with additional vortexing at 5 minute intervals. The samples were then placed in ice for 5 minutes to deactivate the Proteinase K. 150 µL of MPC Protein Precipitation Reagent was then added to each sample and mixed vigorously by vortexing for 10 seconds. Proteins were pelleted from the samples by centrifuging for 10 minutes at 10,000 rpm and 4°C. The supernatants were transferred to clean 1.5 mL microcentrifuge tubes, and the pellets were discarded. Then, 500 µL of 100% isopropanol was added to each recovered supernatant. Each tube was inverted approximately 30 times to gently mix the solution. The nucleic acids were pelleted from the samples by centrifuging each tube for 10 minutes at 13,000 rpm and 4°C. The supernatants were removed and discarded by pouring off the isopropanol. The DNA pellets were rinsed with 1.0 mL of 70% ethanol and centrifuged for 2 minutes at 13,000 rpm and 4°C. The ethanol was removed from the samples by pouring off and vacuum drying the remnant liquid. Finally, the nucleic acids were resuspended in 50 µL of TE buffer and stored at -20°C until analysis by PCR amplification.

For samples EE424-EE809, DNA isolation was achieved by utilizing a CTAB buffer protocol as follows. To each sample tube, a volume of 250 µL lysis buffer was added. A small pestle was used to grind the tick within the tube. Then, 350 µL CTAB buffer [50 mM Tris-HCl pH 8, 4 M NaCl, 1.8% CTAB (hexadecyltrimethyl-ammonium bromide), and 25mM EDTA pH 8] was added to each tube. Each sample was incubated at 65°C for 20 minutes, with 5 minute intervals to vortex and 10 minute intervals to regrind the samples, as necessary. After incubation, a volume of 500 µL Chloroform: isoamyl alcohol (24:1) was transferred to each tube and mixed by vortexing. The tubes were centrifuged at 14,000 rpm for 5 minutes, and the supernatant transferred to a clean

1.5 mL microcentrifuge tube. To each of these tubes, 500 µL of Chloroform was added, mixed gently, and centrifuged for 5 minutes at 14,000 rpm. The supernatant was transferred from each sample to a clean 1.5-mL microcentrifuge tube. Next, 1/10 volume of sodium acetate was transferred to each sample, followed by 1/1 volume of cold 100% isopropanol. The samples were mixed gently and placed at 0°C for 30 minutes to precipitate the DNA. After the incubation period, DNA was pelleted by centrifugation at 14,000 rpm for 15 minutes. Two successive washes of the DNA pellet were performed using 1.0 mL of 70% ethanol and 1.0 mL of 100% ethanol by centrifuging the tubes for 3 minutes and discarding the supernatant. The pellet was vacuum-dried and resuspended in 50 µL of 10mM Tris-HCl buffer.

Sample analysis

PCR Amplification

Direct and nested polymerase chain reactions were used to amplify the 16S rRNA gene and *ank* gene of *A. phagocytophilum.* Amplifications were performed in a MJ PTC 200 Peltier Thermal Cycler or Perkin Elmer GeneAmp PCR System 9600. PCR reaction mixtures were made in an airflow hood that was regularly irradiated with UV light. Additionally, pipettes designated only for operation in the specially designed hood were used to reduce further sources of contamination.

The primary and nested reactions of the 16S rRNA assay used oligonucleotide sequences based on *Anaplasma phagocytophilum* previously described (Barlough et al., 1996). In the primary reaction, the forward primer was EE1: 5’ TCC TGG CTC AGA ACG AAC GCT GGC GGC, and the reverse primer was EE2: 5’ AGT CAC TGA CCC AAC CTT AAA TGG CTG. Each reaction used 2.5 mM magnesium chloride, 0.1 mM

of each dNTP, 0.5 µg of DNA, 0.4 µM of each primer, and 0.625 U *Taq* DNA polymerase (HotStarTaq®, Qiagen, Valencia, CA) in a total volume of 25 µL (Pusterla et al., 1997). The primary reaction program included 15 min at 95°C to activate the *Taq*, 5 min denaturation at 94°C, 35 amplification cycles at 94°C for 1 min and 72°C for 2 min, and a 5 minute elongation at 72°C. The nested reaction used primers EE3: 5' GTC GAA CGG ATT ATT CTT TAT AGC TTG C and EE4: 5' CCC TTC CGT TAA GAA GGA TCT AAT CTC C, as the forward and reverse primers, respectively. Each nested reaction included 1 µL of the 1407-bp product from the primary reaction as the template, 2.5 mM magnesium chloride, 0.1 mM of each dNTP, 0.4 µM of each primer, and 0.625 U *Taq* DNA polymerase (HotStarTaq®, Qiagen, Valencia, CA) in a total volume of 25 µL (Pusterla et al., 1997). The nested reaction program included 15 min at 95°C to activate the *Taq*, 5 min denaturation at 94°C, 35 amplification cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, ending with a 5 minute elongation at 72°C.

The *ank* gene assay was based on the PCR protocols of Massung et al. (2000) and Bjöersdorff et al. (2002) with minor modifications. The primary reaction used the forward primer AQ2F3: 5' GAA GAA ATT ACA ACT CCT GAA G and reverse primer AQ2R2: 5' CAG CCA GAT GCA GTA ACG TG (Bjöersdorff et al., 2002). Each primary reaction included 2.5 mM magnesium chloride, 200 µM of each dNTP, ~1.0 µg of DNA, 0.5 µM of each primer, and 0.625 U *Taq* DNA polymerase (HotStarTaq®, Qiagen, Valencia, CA) in a total volume of 25 µL. The primary reaction program included 15 min at 95°C to activate the *Taq*, 5 min denaturation at 95°C, and 40 amplification cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, followed by a 5 minute elongation at 72°C. The primers used for the nested PCR were AQ2F2: 5'

TTG ACC GCT GAA GCA CTA AC and AQ2R1: 5' ACC ATT TGC TTC TTG AGG AG (Bjöersdorff et al., 2002). Each nested reaction included 1 µL of the 723-bp product from the primary reaction as the template, 2.5 mM magnesium chloride, 200 µM of each dNTP, 0.5 µl of each primer, and 0.625 U *Taq* DNA polymerase (HotStarTaq®, Qiagen, Valencia, CA) in a total volume of 25 µL (Pusterla et al., 1997). The nested reaction program was 15 min at 95°C to activate the *Taq,* 2 min denaturation at 95°C, and 30 cycles of amplification at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, ending with a 5 minute elongation at 72°C.

PCR reaction mixtures were stored at -20°C until analysis by agarose gel electrophoresis. Negative (ddH_20) and positive (DNA from cultured HGA agent supplied by R. F. Massung, CDC, Atlanta, GA) controls were included with each primary and nested reaction to discount contamination and verify proper PCR conditions, respectively. The PCR products were visualized on 1.0% agarose gels containing a 1 kb standard DNA ladder and compared to the positive control's band size. Positive samples were run a second time in order to verify findings. Those samples that produced identical results were counted as positives.

Agarose Gel Electrophoresis

DNA isolated from the tick samples and amplified by PCR was visualized on 1.0% agarose gel. Gels were stained with ethidium bromide by adding it to the electrophoresis buffer [1X TAE (Tris base, glacial acetic acid, 0.5M EDTA (disodium ethylenediaminetetraacetate), pH 8.0)] used in the buffer-agarose mixture and aqueous bath. Gels were run at 80 V for approximately 45 minutes. The banding patterns were

visualized on a UV transilluminator and photographed with the Foto/Analyst® Luminary Cooled Camera.

Data analysis

Confirmed positive samples were analyzed using G-tests for homogeneity to detect prevalence differences among all island and mainland sites, between each classification of the study site, and sample sex (JMP, SAS Institute, Cary, NC).

Randomly chosen positive samples from each site were prepared for sequencing. The *ank* nested PCR products from samples EE222, EE636, EE754, and EE791 and 16S rRNA nested PCR products from sample EE222 were cleaned using the QIAquick PCR purification Kit (Qiagen, Valencia, CA). In addition, the primary reaction products from sample EE222 were purified to ascertain the amplified sequence after elongation from the outer primers. Sequencing was performed with an ABI 3100 Automated Sequencer (Clemson University Genomic Institute, Clemson University). A BLAST search was administered on GenBank to determine which previously reported sequences were homologous to the sequences attained from this study.

CHAPTER 3

RESULTS

Anaplasma phagocytophilum prevalence within *Ixodes scapularis*

Ank gene assay

The results of the *ank* assay, seen in Figure 3.6, were used to determine positive and negative samples. During two collecting seasons, a total of 808 *I. scapularis* ticks were collected from two island and four mainland sites and analyzed. Of all the ticks sampled, 158 were positive for *A. phagocytophilum* with an overall infection rate of 20% (CI=2.76) in the region. The prevalence of *A. phagocytophilum* infected ticks was greatest on both barrier islands supporting feral horse populations. Cumberland Island had the highest prevalence of 23% (CI=3.23); Shackleford Banks populations had an infection rate of 19%. Tick populations from mainland sites in Camden, Bulloch, Chatham, and Glynn Counties had infection prevalence ranging from 17% (CI=15.03) to 0.0%. A summary of the ticks collected from each site can be seen in Table 3.1.

A highly significant difference ($\alpha \leq 0.05$) was found between the combined numbers of infected ticks on island versus mainland sites (G=19.768; df=1; P= <0.0001), which is seen in Figure 3.1. Additionally, a significant difference was found when all the sites were compared (G=26.251; df=5; P=0.0001). However, there was no significant difference, seen in Figure 3.2, between the two island sites (G=0.328; df=1; P=0.5670) or the four mainland sites (G=5.861; df=3; P=0.1186). At the Cumberland Island study site, the large sampling area was divided into regions and zones. A significant difference was found between the 12 zones on the island (G=33.947; df=11; P=0.0004) (Fig. 3.3), and the three regions being north, south, and central (G=10.855; df=2; P=0.0044) (Fig. 3.4).

In addition to analyzing variations among sites, infection variations due to sex differences were examined (Fig. 3.5). Overall males showed significantly different infection prevalence than females (G=5.204; df=1; P=0.0225). At individual locations, significant variations by sex were seen in Camden County (G=5.401; df=1; P=0.0201) and Shackleford Banks (G=12.004; df=1; P=0.0005).

16S rRNA gene assay

The 16S rRNA gene assay had non-specific amplification of DNA sequences. As can be seen in Figure 3.7, the amplified region of the positive control was smaller than the region amplified in samples that were initially interpreted as positive. It was originally hypothesized that more samples would be positive compared to the *ank* assay, which has higher specificity. However, of the 808 ticks sampled, a prevalence of 4% (CI= 1.35) was found in *I. scapularis* infected with *A. phagocytophilum.* Additionally, samples that were positive with the 16S rRNA assay did not always correspond with positive samples of the *ank* assay. Because of the inconsistencies with the *ank* assay and the positive control, this assay was not used for further data analysis.

Sequence analysis of *Anaplasma phagocytophilum* isolates

The results of the BLAST search confirmed that the randomly chosen isolates were *A. phagocytophilum*. The isolates from Chatham County and Cumberland Island showed greatest sequence homology with human and canine isolates from the northeastern United States, most notably Minnesota, Rhode Island, New York, and Wisconsin. Shackleford Banks and Camden county isolates had sequence homology with northeastern variants, in addition to horse isolates from California. The most significant sequence alignments did not include variants of *A. phagocytophilum* from Europe.

CHAPTER 4

DISCUSSION

Prevalence of *A. phagocytophilum* and significance of results

The presence of *A. phagocytophilum*-infected ticks collected from 5 of the 6 study sites provides evidence that the bacteria are not confined to the west and northeast. Perhaps the topic of most interest is how these results compare to previous findings in other biogeographical regions. Studies in the western United States have shown that the prevalence of *A. phagocytophilum* in the vector species *Ixodes pacificus* (6%) and *Dermacentor variabilis* (9%) were markedly lower than those we observed (Holden et al., 2003). In a similar study conducted in 1994, questing *I. scapularis* ticks collected from Nantucket Island had an 11% infection rate (Telford et al., 1996). A decrease in infection rates from 10% to 1% in 1995 and 1996 was observed in a survey of southern coastal Maine (Holman et al., 2004). These representative studies illustrate the variation in prevalence between regions. The observation that infection rates at three of our six collection sites and the cumulative prevalence (20%) are higher than those previously mentioned suggests that the southeastern United States has greater potential as foci for *A. phagocytophilum*. Interestingly, the first accepted cases of HGA were reported from Wisconsin in 1994, but from 1986 to 1988, cases were being reported in the southeastern United States (McDade, 1990). Once again, the potential for *A. phagocytophilum* distribution in the region has gone unstudied for decades and may be underestimated.

In comparison to the one survey conducted in the southeast (Fang et al., 2004), the observed infection rates are much higher. The variability in distribution of the infectious agent on a large and small-scale may be attributed to the host and vector

dynamics at the study site and movement of foci (Foley et al., 2004). For example, in this study Cumberland Island had the highest prevalence of *A. phagocytophilum*-infected ticks. The island has a large wildlife population that is restricted to the 18-mile stretch of land. While deer and other large mammals are prevalent on the island, it has been suggested that they may not be important in the transmission cycle (Keel et al., 1995). The white-tailed deer (*Odocoileus virginianus*) can be naturally infected with *A. phagocytophilum* (Dawson et al., 1994). However, adults are the predominant life-stage of the vector species found on white-tailed deer (Tate et al., 2005) in the region. Therefore, the probability of an immature tick becoming infected with *A. phagocytophilum* from a deer reservoir is low. Instead smaller mammals, such as the cotton mouse (*Peromyscus gossypinus*), are implicated in the transmission and propagation of the bacteria. On the island, large populations of the mammals serve as reservoir hosts for the bacteria, and when an infected immature tick drops, dispersal is limited because of the geographical confines. Thus, the prevalence rates of *A. phagocytophilum* in the area are expected to be higher than at a mainland site, where no limitations are placed on the host or vector.

One would assume that this interaction would also drive the infection rates of other barrier islands, such as Shackleford Banks, to a greater capacity. While ticks collected at this site had a high prevalence (19%), it was not markedly different than the highest prevalence observed at a mainland site, Chatham County (17%). The island is not only smaller than the aforementioned Cumberland Island, but it also has smaller areas of vegetation that facilitate tick questing and fewer reservoir hosts, such as the cotton mouse and white-tailed deer (Telford et al., 1996). The infection rates at mainland sites

follow the same principles, with large undeveloped areas and sizeable host populations facilitating the movement and maintenance of *A. phagocytophilum*. However, since the infected tick or host populations are not restricted to an area, the distribution is broader.

In addition to terrestrial animals facilitating infected-tick dispersal, migratory birds have also been implicated. The most widely accepted theory is that the birds are mere occasional or mechanical hosts (Hubálek, 2004), transferring the infected ticks from one site to another. However, robins have been shown to maintain the EGA and HGA agent, acting as competent reservoir hosts (Daniels et al., 2002). Both scenarios would increase the distribution of *A. phagocytophilum* and explain the trends seen in the United States, which we can conclude based on the results of this and previous studies.

While infection rates between male and female *I. scapularis* ticks were significant, vector gender probably has little effect on transmission in the region. Male ticks do not fully engorge and feed only intermittently (Oliver, 1989). Thus, the acquisition of the infectious agent must occur during the immature stages when the tick fully engorges. However, the male may take blood from a female during the mating period. Evidence of this parasitic feeding is scarring seen on female ticks (J.H. Oliver, personal communication, June 19, 2006). Since the adult male will often mate an average of 2 to 3 times (Sonenshine, 1993), the chances to acquire the bacterium during its life cycle is increased compared to the adult female, who feeds and mates once. The probability of encountering infected male ticks, however, is random. Thus, the significant gender difference findings of this study are not scientifically considered.

With the potential for widespread *A. phagocytophilum* maintenance and transmission, it can be inferred that the feral and domestic horse populations in and near

the collection sites are at risk for infection, as well as any other mammals in the area. The risks of infection for domestic horses may be lower because of mobility of the agent in the region and the regular care of the horses. Conversely, the high prevalence rate on the barrier islands suggests feral horses are highly susceptible, possibly due to greater exposure to infected ticks. On Cumberland Island, there is the greatest potential for transmission and maintenance of the EGA causative agent. Although direct PCR or serology has not been performed on blood samples from these horses, some evidence of illness with symptoms of anorexia and lethargy has been noted from personal observations. It would be beneficial to conduct such an investigation to ascertain the prevalence of EGA cases within the herd, or any other infectious agent that causes these common symptoms. The herd at Shackleford Banks is already monitored for Equine Infectious Anemia and reproductive control by the responsible department; and additional testing could also be performed to determine EGA prevalence. The horses on Cumberland Island, however, are not monitored.

The herds on Shackleford Banks and Cumberland Island are not native, and thus, some debate exists on whether or not the health of the horses should be preserved. If EGA was prevalent in the horse population, it could act as natural population management of a species that destroys native flora and fauna. However, if the maintenance of *A. phagocytophilum* on the island was facilitated by its presence within the equine population, the risk for infection to humans visiting the island would greatly increase, increasing public health risks.

Sequence analysis of *Anaplasma phagocytophilum* isolates

The sequence alignment of the agent can provide data that may contribute to our understanding of the ecology of *A. phagocytophilum*. The same can be said for any variant identified in the feral and/or domestic horse populations from future studies.

The positive PCR products from the *ank* assay were compared to sequences from the western and northeastern United States and areas of Europe. Because the 16S rRNA gene assay did not successfully amplify *A. phagocytophilum*, comparisons to less phylogenetically diverse organisms could not be performed. The sequencing and PCR results showed that the primers were not specific. We suspect that the error occurred during the primary reaction, where the melting temperatures of the forward and reverse primer varied more than 7°C. In the future, we would like to attempt another 16S rRNA assay using different primers. The sequence of these positive samples would provide additional insight into the evolution and distribution of *A. phagocytophilum*.

The results of the *ank* assay indicate that the isolates from this region of the United States share sequence homology with isolates from other regions, such as the northeastern and western United States. The movement of the infectious agent throughout the country via reservoir hosts may explain how these regionally different isolates can share homology. If reservoir hosts move the bacteria in a wave-like pattern across the states, we would expect to find *A. phagocytophilum* that is genetically similar throughout. Our findings support this, suggesting that reservoir hosts are instrumental in the propagation and movement of the bacteria. Reservoir and mechanical hosts with a wide range, such as migratory birds, are most likely the greatest source of the bacteria's movement. The homologous sequences are also from a variety of organisms, including

humans, dogs, and horses. Aligning genetically similar isolates from different animals further supports the proposal that the HGA and EGA agent are the same species, *A. phagocytophilum*.

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Table 3.1. Prevalence of *Anaplasma phagocytophilum* in adult *Ixodes scapularis* ticks at barrier island and mainland study sites in the southeastern United States.

Collection Site	Sex	Sampled	Infected	% Infected
Cumberland Island, GA	Female	366	74	20
	Male	267	67	25
	Total	*633*	*141*	*22*
Shackleford Banks, NC	Female	21	0	0
	Male	22	8	36
	Total	*43*	*8*	*19*
Bulloch County, GA	Female	22	0	0
	Male	22	1	5
	Total	*44*	*1*	*2*
Camden County, GA	Female	26	0	0
	Male	29	4	13
	Total	*55*	*4*	*7*
Chatham County, GA	Female	11	1	9
	Male	13	3	23
	Total	*24*	*4*	*17*
Glynn County, GA	Female	3	0	0
	Male	6	0	0
	Total	*9*	*0*	*0*

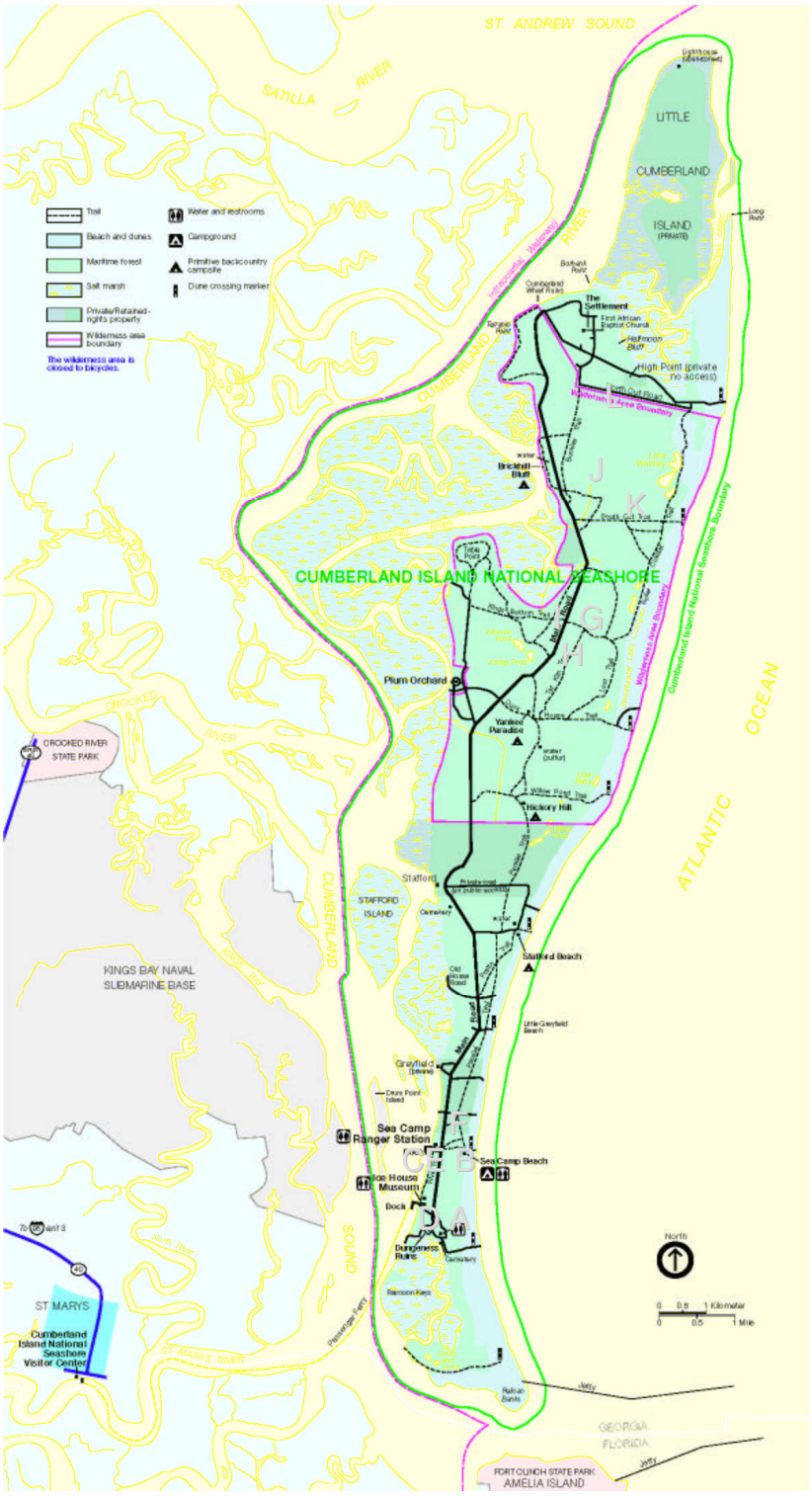

Figure 2.1. Cumberland Island, Georgia collection site. The map indicates the zones where ixodid tick samples were collected from the island. Zones A-F are at the southern end; zones G-I are at the center of the island; zones J-L are at the northern end.

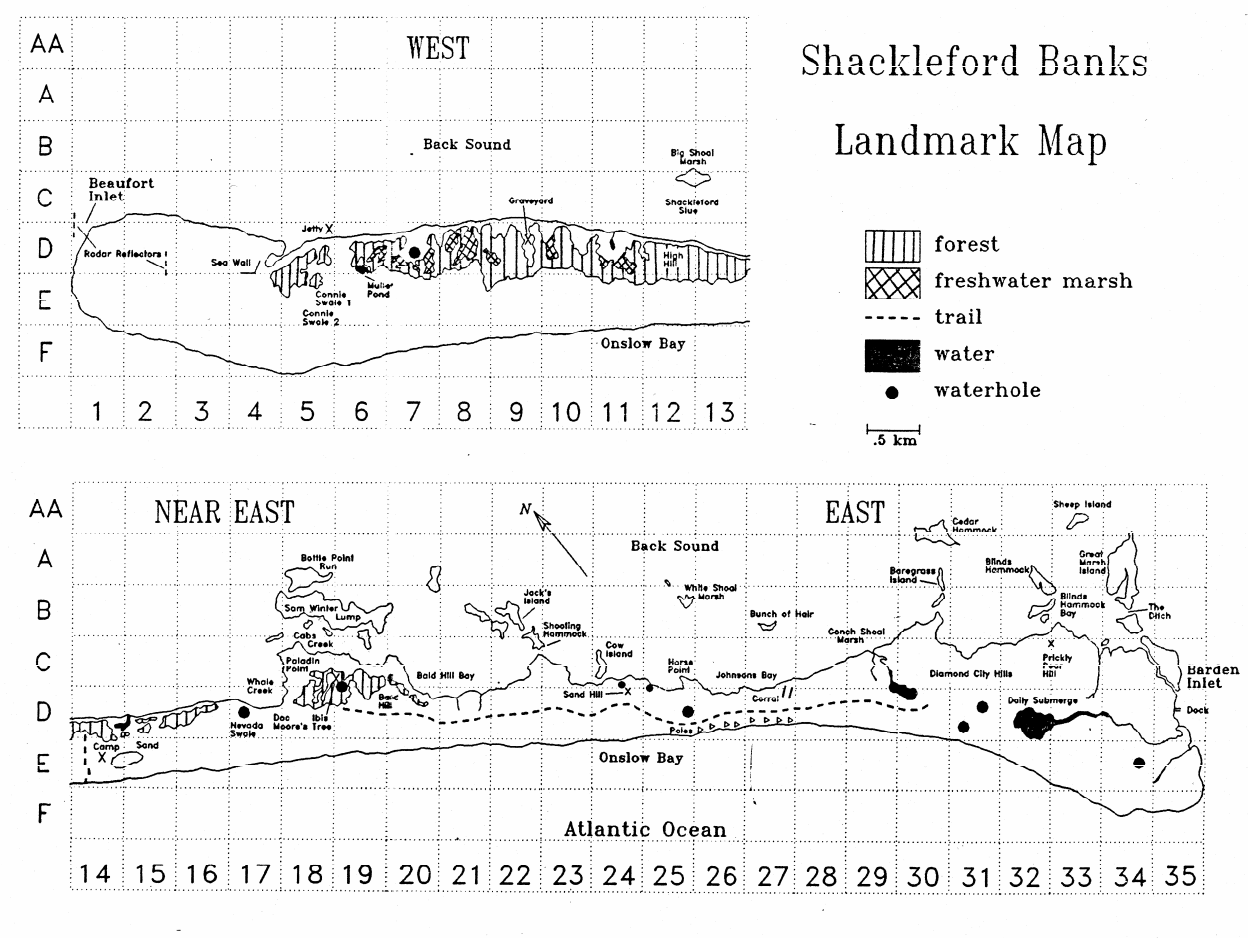

Figure 2.2. Cape Lookout, North Carolina collection site. The map highlights the location of the forest on the island's western end, where ixodid tick samples were collected.

Figure 2.3. Camden County, Georgia collection site**.** The asterisk indicates the approximate location in the satellite image where ixodid ticks were collected.

Figure 2.4. Bulloch County, Georgia collection site. The asterisk indicates the approximate location in the satellite image where ixodid ticks were collected.

Figure 2.5. Chatham County, Georgia collection site. The asterisk indicates the approximate location in the satellite image where ixodid ticks were collected.

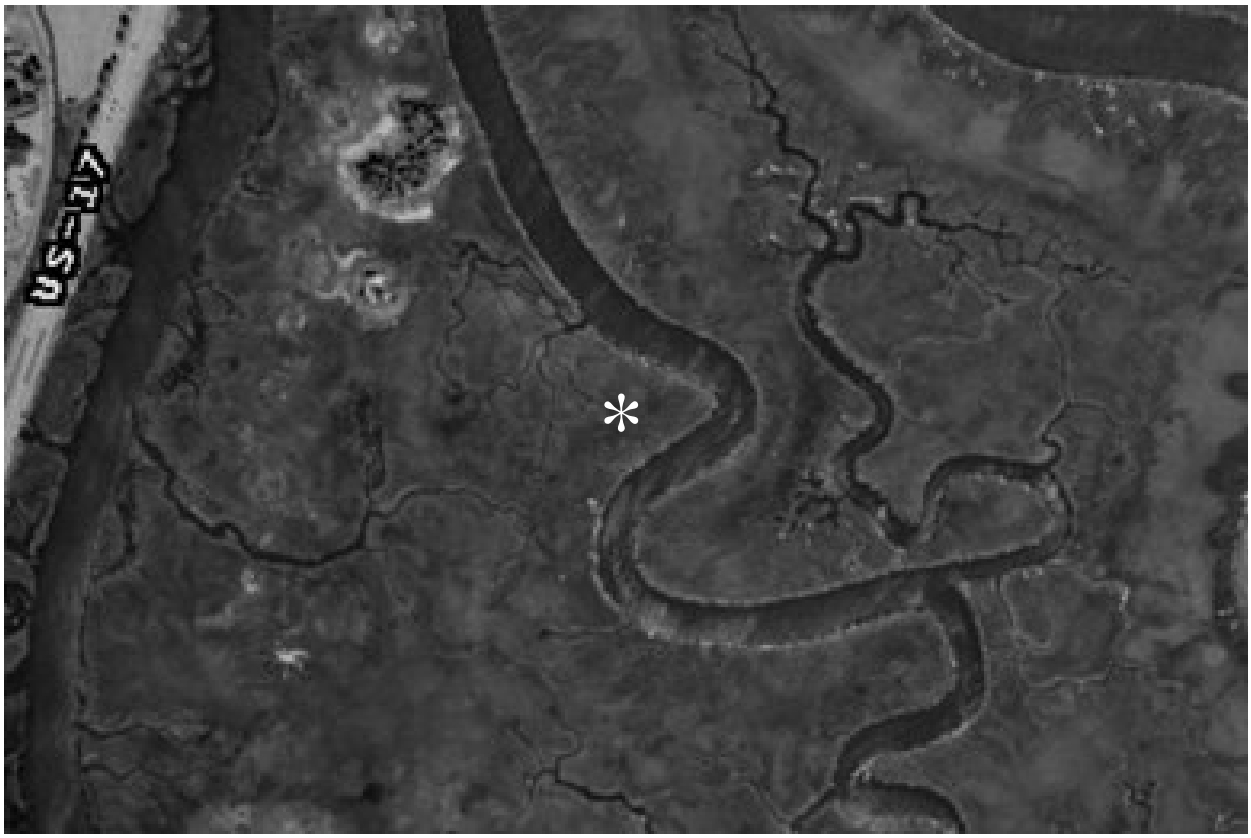

Figure 2.6. Glynn County, Georgia collection site. The asterisk indicates the approximate location in the satellite image where ixodid ticks were collected.

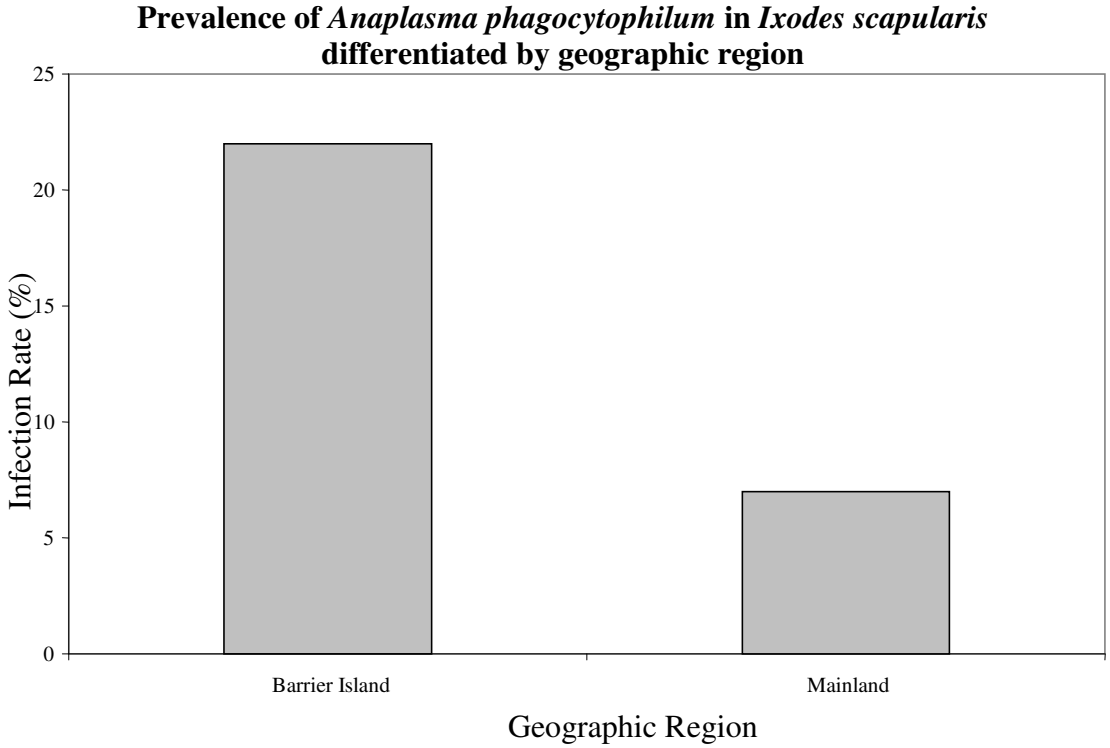

Figure 3.1. Prevalence of *Anaplasma phagocytophilum* in *Ixodes scapularis* differentiated by geographic region. Data shows a significant difference ($\alpha \leq 0.05$) between adult ticks collected at barrier island sites with feral horses than mainland sites with domestic horses ($G=19.768$; $df=1$; $P= < 0.0001$).

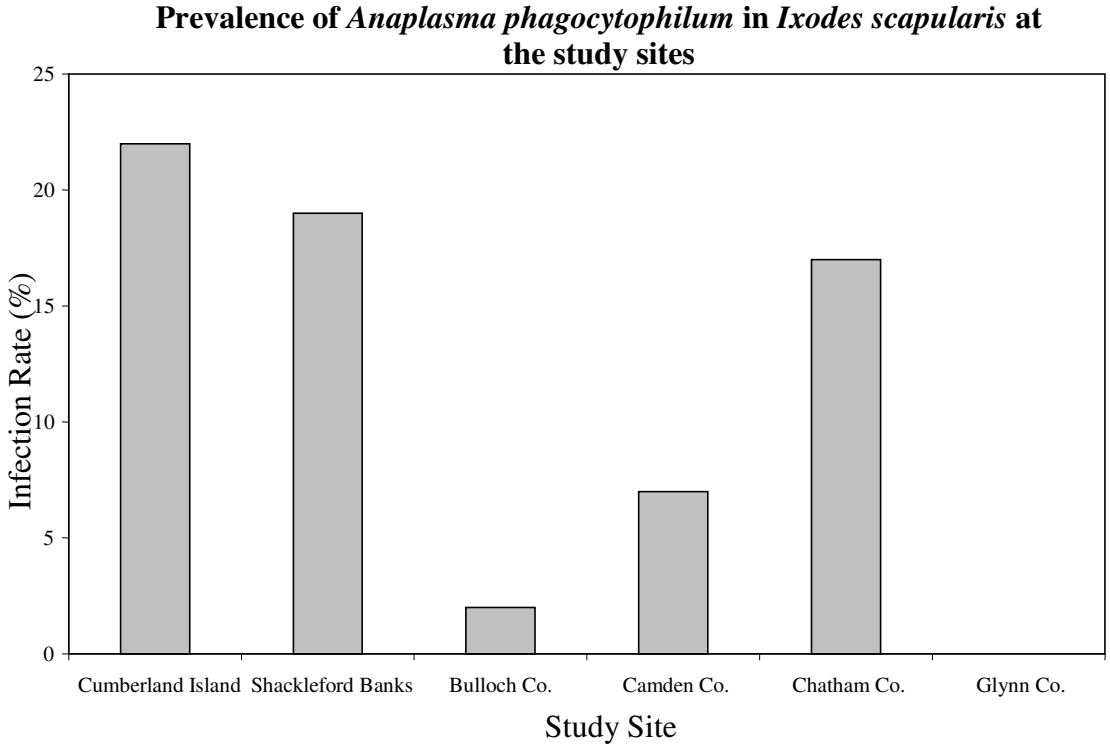

Figure 3.2. Prevalence of *Anaplasma phagocytophilum* in *Ixodes scapularis* at the study sites. Data indicates a significant difference ($\alpha \leq 0.05$) between the infection rates at the 6 study sites (G=26.251; df=5; P=0.0001).

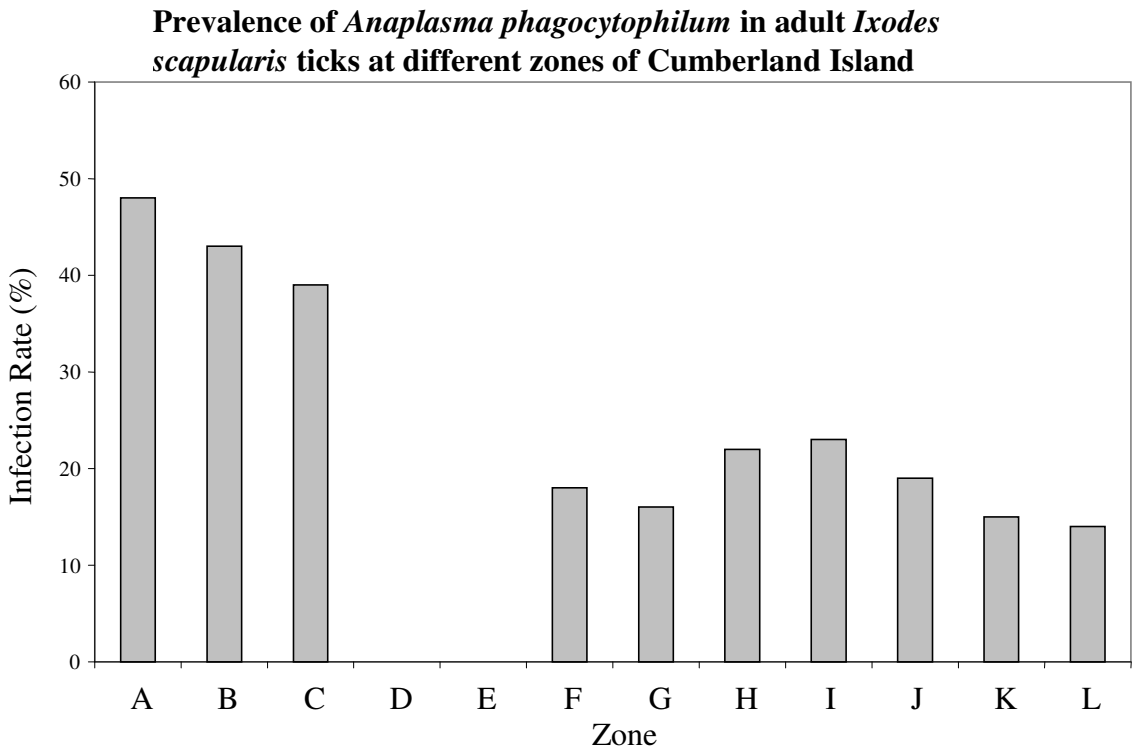

Figure 3.3. Prevalence of *Anaplasma phagocytophilum* in adult *Ixodes scapularis* ticks at different zones of Cumberland Island. Data shows a significant difference ($\alpha \leq 0.05$) between the 12 collecting zones on the barrier island (G=33.947; df=11; P=0.0004).

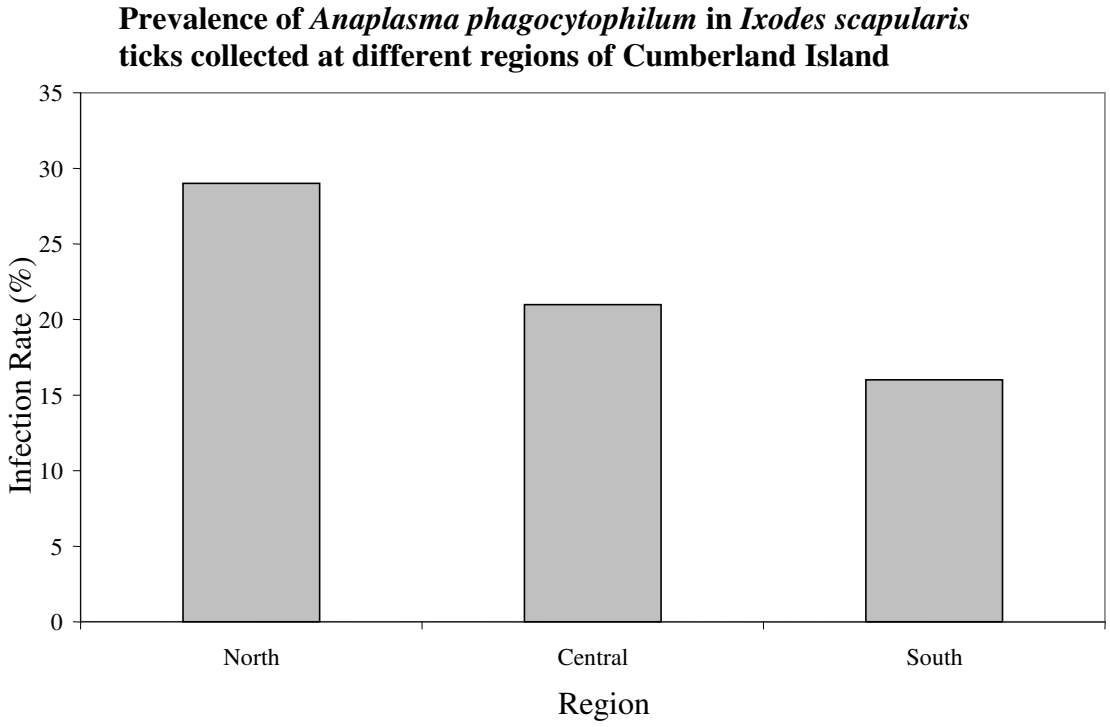

Figure 3.4. Prevalence of *Anaplasma phagocytophilum* in *Ixodes scapularis* ticks collected at different regions of Cumberland Island. A significant difference ($\alpha \leq 0.05$) was found between the three geographic regions of the island (G=10.855; df=2; P=0.0044).

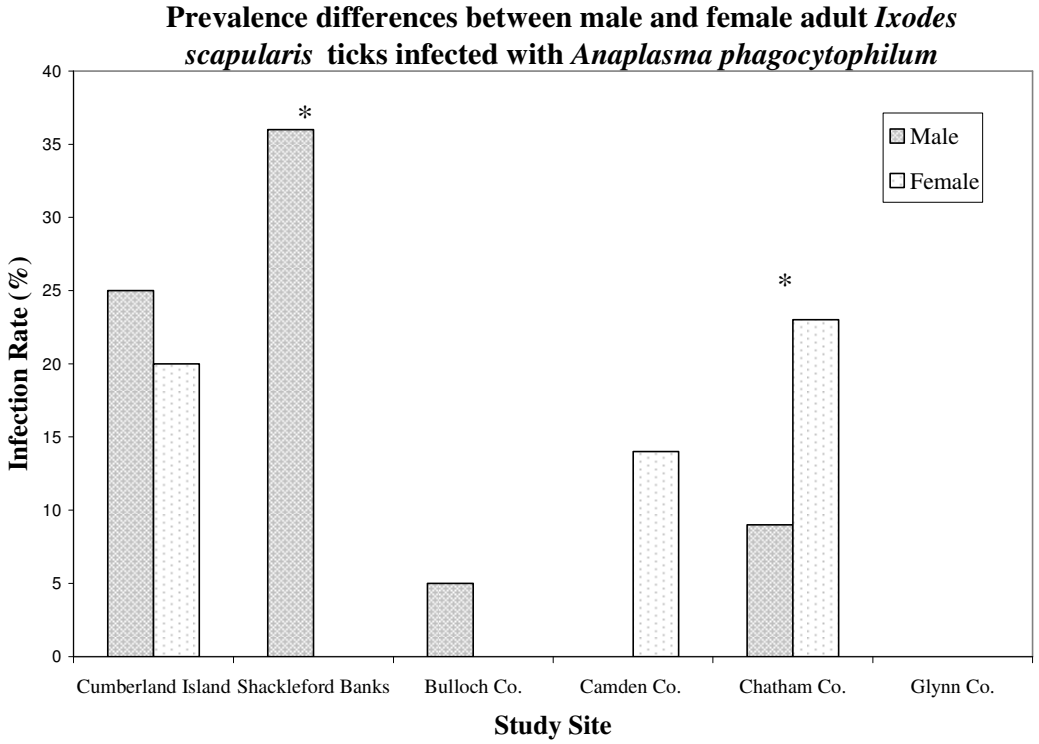

Figure 3.5. Prevalence differences between male and female adult *Ixodes scapularis* ticks infected with *Anaplasma phagocytophilum*. Significant differences ($\alpha \leq 0.05$) between the two sexes were observed at Camden County (G=5.401; df=1; P=0.0201) and Shackleford Banks (G=12.004; df=1; P=0.0005).
*Significance between sexes at individual study sites

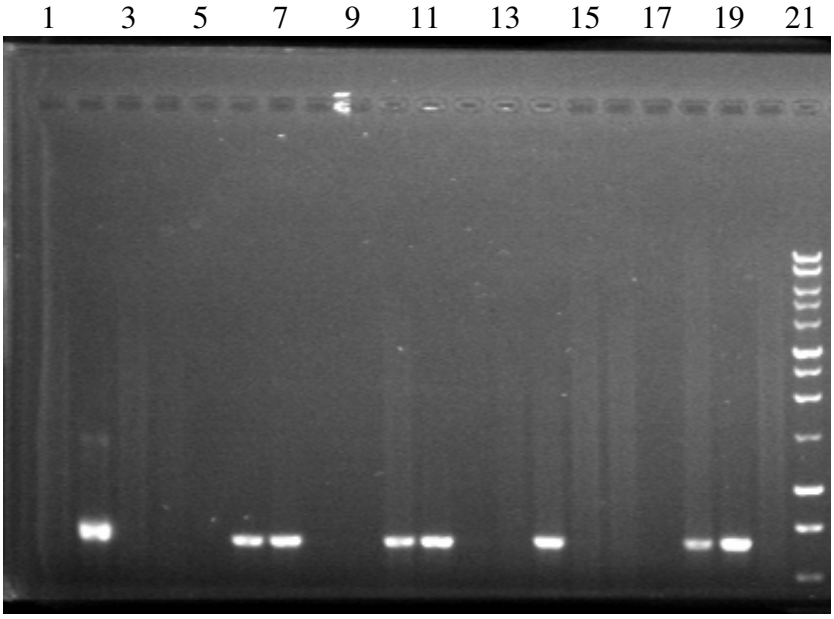

Figure 3.6. Agarose gel of *ank* gene nested PCR amplification from *I. scapularis* ticks. Lane 1: negative control. Lane 2: positive control. Lanes 6, 7, 10, 11, 14, 18, 19: positive samples. Lane 21: 1 kb ladder.

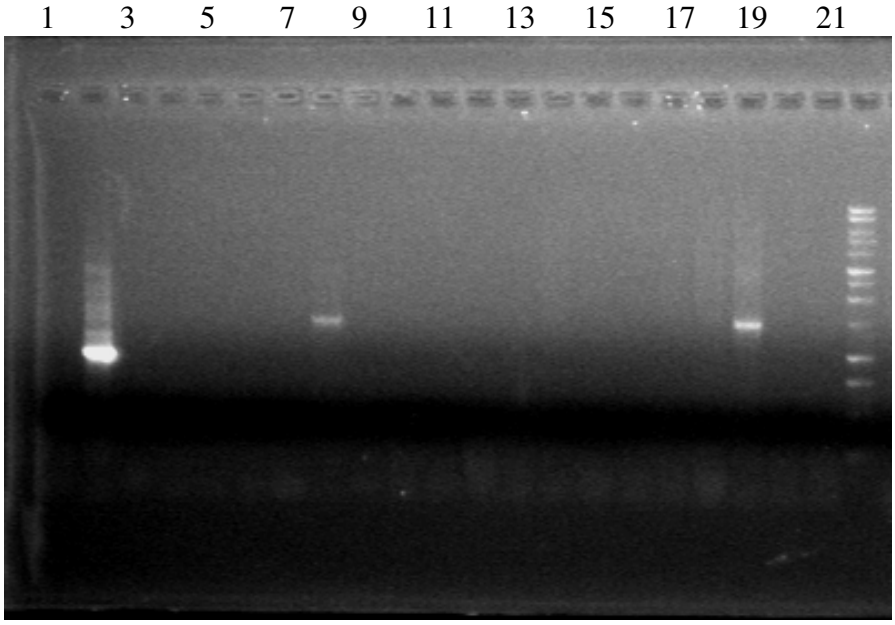

Figure 3.7. Agarose gel of 16S rRNA gene nested PCR amplification from *I. scapularis* ticks. Lane 1: negative control. Lane 2: positive control. Lanes 8, 19: presumed positive samples. Lane 22: 1 kb ladder.